

Amendments to the Specification:

Please replace the paragraph on page 7, beginning line 12, with the following amended paragraph:

The Sequence homology between Der p 3 and bovine trypsin are shown below. Residues signalled with * are implicated in the catalytic site.

Der p 3	1	MIYNTILIV	MAINTILNP	ILASPNAI	VGGEKALAGE	CPYQSLQSS
Trypsine	1	-----MHPI	ILALFVGAAY	AFESDDDKI	VGGYTCAENS	VPYQSLNAG
				*		
Der p 3	51	SHFCGCSILE	EYWEIAAHC	VAGQTASKIS	IRYNLKHSL	GCEKISVAKI
Trypsine	51	YHFCGCSILE	QWVISAHC	YQYHIQVETG	PYRIDVLEG	GEQFIDASKI
				*		
Der p 3	101	FAHKKVLSQ	INDIALIKL	KSPMKINDEN	AAVGLPAKG	SDRVGDDQVR
Trypsine	101	IRHKKVSSNT	INDILLIKL	FAVEN---	AVSTLLIPS	ACASRTECL
				*		
Der p 3	151	MSGNGYLEEG	SYSIPSEIRR	VDIAYSRKE	CNELGSKANA	EVINDNMICGG
Trypsine	151	MSGNGTLLSS	GVNIEDLIQC	IVAPILLSHAD	CEASHP----	CQNTNMICRG
				*		
Der p 3	201	DVANGGKDSQ	QDSGGGVVD	VNNQVVGIV	SWGYGCAKRG	YPGVYTSVGN
Trypsine	201	FLEGGKDSQ	QDSGGGVAC	NG--CLQGIV	SWGYGCAQKG	KPGVYTEVGN
				*		
Der p 3	251	NDWIESKRS	Q--	(SEQ ID NO:58)		
Trypsine	251	NDWIDETIA	ANS	(SEQ ID NO:59)		

Please replace the paragraph on page 13, beginning line 11, with the following amended paragraph:

A further aspect of the present invention provides an isolated nucleic acid encoding a mutated version of the Der p 1/ProDer p 1/PreProDer p 1 allergen as disclosed herein. Preferably the nucleotide sequence is a DNA sequence and can be synthesized by standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts *et al* in Biochemistry 1985, 24, 5090-5098, by chemical synthesis, by *in vitro* enzymatic polymerization, or by a combination of these techniques. Preferably the nucleic acid sequence has a codon usage pattern that has been optimised so as to mimic the one used in the intended expression host, more preferably resembling that of highly expressed mammalian e.g. human genes. Preferred DNA sequences are codon-optimised sequences and are set out in SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, ~~SEQ ID NO:15 and SEQ ID NO:17~~ SEQ ID NO:16 and SEQ ID NO:18.

Please replace the paragraph on page 35, beginning line 4, with the following amended paragraph:

2. – Site-directed mutagenesis

Mutagenesis of Der p 1 cysteine residues at position 4, 31 or 65 (mature ProDer p 1 numbering, corresponds to positions 84, 111 or 145 in ProDer p 1) was performed in the plasmid pNIV4854, after the substitution of DNA fragments carrying one of the three cysteine codons by synthetic oligonucleotides containing the mutations. The following oligonucleotides were used:

5'TTAAGACCCAGTTTGATCTCAACGCGGAGACCAACGCCCGTATCAACGGCAAT
GCCCCCGCTGAGATTGATCTGCGCCAGATGAGGACCGTGACTCCCATCCGATGC
3' (forward) (SEQ ID NO:27) and 5'CGGATGGGAGTCACGGTCCTCATCTG
GCGCAGATCAATCTCAGCGGGGGCATTGCCGTTGATACTACGGGCGTTGGTCTCC
GCGTTGAGATCGAAACTGGGTC3' (reverse) (SEQ ID NO:28) to generate a 110bp *Afl* II-
Sph I fragment for the mutation of cysteine residue 4 to arginine (C4R),
5'CAAGGCGGCCGCTGGGTCTTGTGGGCCTTTTCAGGCGTGCCGCGACAGAGTC
GGCATACCTCGCGTATCGGAATCAGAGCCTGGACCTCGC3' (forward) (SEQ ID
NO:29) and
5'TCAGCGAGGTCCAGGCTCTGATTCCGATACGCGAGGTATGCCGACTCTGTGCG
GCCACGCCTGAAAAGGCCAACAAGACCCACGGCCGCCTTGCATG3' (reverse)
(SEQ ID NO:30) to generate a 98bp *Sph* I-*Bsp* I fragment for the mutation of cysteine residue
31 to arginine (C31R), 5'TGAGCAGGAGCTCGTTGACCGTGCTCTCC
CAACACGGATGTCATGGGGATACGATTCCCAGAGGTATCGAATACATCCAGCAT
A3' (forward) (SEQ ID NO:31) and 5'CTGGATGTATTCGATACCTCTGGGAATCGTAT
CC CCCATGACATCCGTGTTGGGAGGCACGGTCAACGCGCTCCTGC3' (reverse)
(SEQ ID NO:32) to generate a 82bp *Afl* II-*Sph* I fragment for the mutation of cysteine residue
65 to arginine (C65R).

Please replace the paragraph on page 39, beginning line 30, with the following amended paragraph:

Primer1 : 5'-GCTATTACCGA[TACGTA]GCTAGGG-3' (SEQ ID NO:50)

This primer comprises the *Sna*BI restriction site downstream of the zone to be deleted.

Please replace the paragraphs on page 40, beginning line 30, with the following amended paragraphs:

Primer2 : 5'-CCGTTGTCGCGATCCTTGATTCCGATGATGACAGCG-3' (SEQ ID NO:51)

This primer is therefore homologous to part of the ProDer p 1 sequence, that downstream and upstream of the zone to be deleted.

Primer3 :
5'-CGGAATCAAGGATCGCGACAACGGGATATCAGCCAACTACC-3' (SEQ ID NO:52)

This primer is also homologous to part of the ProDer p 1 sequence and will also allow deletion of 42pb. In addition, it contains a point mutation which will make it possible to modify the EcoRV site.

Primer4 : 5'-TAGGGGAGCTCAGATCTGATCCACTGAC-3' (SEQ ID NO:53)

Please replace the paragraphs on page 41, beginning line 1, with the following amended paragraphs:

662 *SnaBI* GCTATTACCGATACGTAAGCTAGGG → (SEQ ID NO:50)

← GCGACAGTAGTAGCCTTAGTTCCTA GCGCTGTTGCC 867 (SEQ ID NO:51)

42pb

3' terminal

825 CGGAATCAAGGAT CGCGACAACGG G TATCAGCCAACTACC → (SEQ ID NO:52)

mutation

42pb

← TAGGGGAGCTCAGATCT 1076 (SEQ ID NO:54)

GGATCC *AvrII* (SEQ ID NO:55)

ACT

GAC

Final PCR

Hybridisation

662 *SnaBI* → CGCTGTCATCAT CGGAATCAAGGATCGCGACAACGG (SEQ ID NO:56)

← GCGCTGTTGCC CATAGTCGGTTGATGG *AvrII* 1076 (SEQ ID NO:57)

Please replace the paragraphs on page 42, beginning line 23, with the following amended paragraphs:

1. Construction of a PreProDer p 3 synthetic cDNA

A PreProDer p 3 cDNA was synthesised using a set of 10 partially overlapping oligonucleotides. These primers were designed, based on the codon preference of highly expressed E. Coli bacterial genes, and produced by a 394 DNA/RNA Applied Biosystem synthesizer. The degenerately encoded amino acids were not encoded by the most prevalent codons but taking the frequencies of the individual codons into account. For example, AAG or AAA encodes the lysine residue with a respective frequency of 21.45% and 78.55% in highly expressed E. Coli bacterial genes. Consequently, we attempted to follow the same codon frequency instead of selecting only the AAA codon for each lysine residue in the synthetic PreProDer p 3. The oligonucleotides were the following:

5'TCATGATCATCTACAACATTCTGATCGTACTCCTGCTGGCCATTAACTTTGGC
TAATCCGATCTGCCGGCATCCCCGAACGCGACCATCGTTGGC 3' (oligo 1, coding)
(SEQ ID NO:34)

5'CACCACAGAAGTGGCTACTAGACTGCAGGGAGATCTGATATGGGCACTACCA
GCCAGTGCTTTTCGCCGCCAACGATGGTCGCG 3' (oligo 2, noncoding) (SEQ ID
NO:35)

5'GTAGCCACTTCTGTGGTGGTACTATTCTTGACGAATACTGGATCCTGACCGC
GGCACACTGCGTGGCCGGCCAAACAGCGAGCAAACCTCTCC 3' (oligo 3, coding)
(SEQ ID NO:36)

5'GTCGATCTGGTAGCTATCATATTTTTCATGTGCGAAAATTTAGCAACAGAGAT
CTTTTCGCCACCCAGTGAGTGTTCAGGCTGTTGTAACGAATGGAGAGTTTGCTC
GCTG 3' (oligo 4, noncoding) (SEQ ID NO:37)

5'GATAGCTACCAGATCGACAATGACATTGCGCTGATCAAGCTGAAATCCCCTAT
GAAGCTGAACCAGAAAAACGCCAAAGCTGTGGGCCTGC 3' (oligo 5, coding) (SEQ
ID NO:38)

5'GATGCATGCTCGAGCGGC3' (oligo 12, noncoding) (SEQ ID NO:47).

Please replace the paragraph on page 45, beginning line 5, with the following amended paragraph:

The deletion of the putative Der p 3 signal peptide was performed by PCR and using two new primers: 5'CATATGAATCCGATCCTGCCGGCATCCCC3' (oligo 13, coding) (SEQ ID NO:48) and 5'GGATCCTCACTGGCTACGTTTAGATTCAATCC3' (oligo 14, non coding) (SEQ ID NO:49). Amplification of the ProDer p 3 cDNA was done by PCR with Taq Polymerase (Roche Diagnostics), 15 cycles: denaturation at 97°C for 30 s, annealing at 65°C for 30 s and elongation at 72°C for 1 min. The resulting 750bp fragment was cloned into a pCRII-TOPO cloning vector (Invitrogen). Top 10 competent E Coli were transformed by the resulting plasmid. 9 clones appeared positively inserted; digestion with *EcoRI* proved clones 1,4,8 to be correctly inserted, while sequencing showed that only clone 4 had the right sequence. The ProDer p 3 cDNA was isolated after the digestions with *NdeI* and *XhoI* and cloned into pET15b digested by the same enzymes. The BL21 and BL21 Star E Coli (Invitrogen) strains were transformed by the resulting plasmid. Addition of IPTG in the culture medium induced the expression of recombinant ProDer p 3 carrying (His)₆ tag at its N-terminal end.

Please replace the paragraph on page 62, beginning line 26, with the following amended paragraph:

The ProDer p 1 coding cassette from pNIV4846 (full-length 1-302aa ProDer p 1 cDNA with optimised mammalian codon usage) was amplified by PCR using the following primers: 5'ACTGACAGGCCTCGGCCGAGCTCCATTAA3' (*StuI* restriction site in bold, forward) (SEQ ID NO:33) and 5'CAGTCACCTAGGTCTAGACTC GAGGGGAT3' (*AvrII* restriction site in bold, reverse) (SEQ ID NO:22). The amplified fragment was cloned into the pCR2.1 TOPO cloning vector. The correct ProDer p 1 cassette was verified by DNA sequencing. Recombinant TOPO vector was digested with *StuI-AvrII* to generate a 918bp fragment which was introduced into the pPIC9K expression vector restricted with *SnaBI*-

AvrII. The resulting plasmid, pNIV4878, contains the ProDer p 1 cassette downstream to the *S.cerevisiae* α factor.

Please replace the paragraphs on page 63, beginning line 6, with the following amended paragraph:

Expression plasmid for the production of unglycosylated ProDer p 1 (N52Q, mature Der p 1 numbering) was derived from pNIV4878 by overlap extension PCR using a set of four primers. The following primers:

5'GGCTTTCGAACACCTTAAGACCCAG3' (primer 1, *Afl*III restriction site in bold, forward) ([SEQ ID NO:23](#)) and 5'GCTCCCTAGCTACGTA TCGGTAATAGC3' (primer 2, *Sna*BI restriction site in bold, reverse) ([SEQ ID NO:24](#)) were used to amplify a 317bp fragment encoding the ProDer p 1 amino acid sequence 71-176.

The following primers 5'CCTCGGTATCGGCAACAGAGCTGGACC3' (primer 3, mutation N52Q in bold, forward) ([SEQ ID NO:25](#)) and 5'GGTCCAGGCTCTGTTGCC GATACGCGAGG3' (primer 4, mutation N52Q in bold, reverse) ([SEQ ID NO:26](#)) were used to introduce mutation N52Q in the ProDer p 1 sequence.